A PRELIMINARY STUDY ON THE OCCURRENCE OF PHENOL-DEGRADING BACTERIA IN CANAGAGIGUE CREEK

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A PRELIMINARY STUDY ON THE OCCURRENCE OF PHENOL-DEGRADING BACTERIA IN CANAGAGIGUE CREEK

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SUMMARY

The occurrence and concentration of phenol-degrading bacteria were examined in the Canagagigue Creek near Elmira, Ontario. The results of microbiological analysis suggested the potential presence of these organisms. However, with respect to the actual degradation of phenols, data from chemical analysis of the inoculated assay medium did not support microbiological observations.

The detection of fluorescent pseudomonads (a groups of aerobic bacteria which are capable of utilizing phenols and other related compounds) and high levels of heterotrophic bacteria are indicative of substantial organic enrichment in this stretch of the Canagagigue Creek.

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INTRODUCTION

Pollution of surface waters with phenol and related compounds has increased consdiderably due to extensive use of these chemicals in various industrial processes (Biczysko and Suschka 1966, Cobb et al. 1975). Both biological and chemical methods have been used for the degradation of phenolic wastes to prevent and decrease contamination of receiving waters. However, due to limited degree of success achieved with these methods, a potentially serious pollution problem exist in waters containing phenolic compounds. The problem is further complicated by the relatively stable and toxic nature of these chemicals. Moreover, only a small group of bacteria and fungi can degrade phenols if the concentration does not exceed 2 g per liter (Iturriaga and Rheinheimer 1972, Rheinheimer 1974).

It is generally assumed that waters contaminated with phenolic compounds represent a major pollution problem only when phenol-degrading bacteria (PDB) are absent. The problem is less significant, however, if relatively large populations of PDB are present (Stafford and Callely 1973). In addition, Iturriaga and Rheinheimer (1972) showed that although such organisms are present in polluted waters both in the spring and winter, the microbial degradation of phenol occurs mainly at water temperatures greater than $10^{o}C$ during the warm season.

The detection and enumeration of PDB is inefficient and difficult by the presently available methods. The procedure generally used involves plating of water and wastewater samples on agar media, transfer of isolated and representative colonies to liquid media containing phenols, incubation at $35^{o}C$ for up to 72 h and chemical determination of undegraded phenol. Population estimate is obtained by counting all colonies, on the original plate, which are similar to the phenol degraders (Ralston and Vela 1974). Phenol degradation in a liquid medium can also be ascertained by growth of PDB and an accompanying change in pH value (Ralston and Vela 1974).

As a part of the waste assimilation study on the Canagagigue Creek (near Elmira Sewage Treatment Plant), a two-day microbiological survey was conducted to determine the occurrence, distribution and concentration of phenol-degrading bacteria (PDB) in water receiving phenolic wastes. The survey was requested by the Water Modelling Section, Water Resources Branch, Ministry of the Environment.

MATERIALS AND METHODS

Sample Collection and Stations

The location of sampling stations in Canagagigue Creek and one of its tributaries (viz. Shirtfactory Creek) is shown in Figure 1. These stations were situated at a variable distance from each other. Further details regarding the exact locations and surrounding areas of these stations are given in Table 1.

On August 24 and 25, 1977, surface water samples were collected in sterile, screw-capped, glass sampling bottles (ca. 175 ml). Effluent samples from the Elmira STP were collected in similar bottles containing sodium thiosulfate to neutralize residual chlorine. The samples were stored on ice for transport to the laboratory. The elapsed time between sampling and analysis was never greater than 18 h in all cases.

Microbiological Analyses

Phenol-Degrading Bacteria (PDB)

The detection and enumeration of PDB were made by the most probably number (MPN) technique using a three dilution-three tube series of a modified medium originally described by Ralston and Vela (1974). The assay medium consisted of the following ingredients:

Solution A (g/l): $NaHCO_3$, 12.5; KH_2PO_4, 10.0; NH_4Cl, 7.0; Na_2SiO_3, 2.0; $FeSO_4 \cdot 7H_2O$, 1.0; $MnCl_2 \cdot 4H_2O$, 0.7; $ZnSO_4 \cdot 7H_2O$, 0.15; phenol red, 2.0; distilled water, 1000 ml.

Solution B: Vitamin-free casamino acid, 0.1 g; distilled water, 100 ml.

Solution C: Phenol, 0.5 g; distilled water, 100 ml.

Experimental Procedure

Ten ml of Solution A was added to 980 ml distilled water (for double strength medium 20 ml of Solution A was mixed with 960 ml distilled water). The pH value was adjusted to 6.85, and autoclaved for 15 minutes at 121°C. After autoclaving, 10 ml (20 ml for the double strength medium) of filter sterilized Solution B was aseptically added to obtain a mixture with a final pH of 8.0.

Filter sterilized Solution C was added in 0.8 ml aliquots to 25 x 150 screw-capped tubes containing 10 ml of double strength A and B mixture. These tubes were then inoculated with 10 ml aliquots of water samples to provide a final volume of 20.8 ml per tube.

Filter sterilized Solution C was added in 0.4 ml aliquots to 13 x 100 screw-capped tubes containing 9 ml of single strength A and B mixture. The tubes were then inoculated with 1 ml of water samples to yield a final volume of 10.4 ml per tube.

In all cases, after inoculation the final concentration of phenol in each tube was approximately 200,000 ppb. The assay medium with all the ingredients was scarlet red in color and remained so on standing and storage at 4°C. Uninoculated tubes containing either single or double strength media with appropriate amount of phenol were maintained as controls. All inoculated and uninoculated (control) tubes were incubated at 35°C for 72 to 96 h. After incubation, the tubes were examined for growth and change (resulting from pH shift) in the color of the medium. All tubes showing no change in color (i.e. remained red) were considered negative. Those tubes in which the color of the medium changed to yellow were scored positive, and were used in estimating the densities (MPN/100 ml) of PDB.

Fluorescent Pseudomonads (FP)

The fluorescent pseudomonads group include Pseudomonas aeruginosa, P. fluorescens and P. putida (Fiest and Hegeman 1969, Stainer et al. 1966). This group of organisms were enumerated by the MPN technique using a three dilution-three tube series of modifed Drake's medium (Drake 1966). The inoculated and uninoculated control tubes were incubated at $41.5 \pm 0.5^{o}C$ for 48 h. Following incubation, the tubes were examined under long-wave ultraviolet light and those tubes showing greenish fluorescent pigment were considered positive. The densities of FP (MPN/100 ml) were ascertained using Ministry of the Environment's MPN tables.

Heterotrophic Bacteria (HB)

Heterotrophic bacterial densities were determined by the spread plate technique using a modified Foot and Taylor medium containing 0.05% peptone and 100 ppm actidione (Young 1977). Water sample and/or appropriate sample dilutions were plated in one ml aliquots using the Fisher Rota-Plate Inoculating Turntable. Three plates were inoculated for each sample/dilution and incubated at $20^{o}C$ for 7 days. The developing bacterial colonies were counted with the aid of a Quebec Colony counter and the densities were expressed as count per ml.

RESULTS

The results of microbiological analyses are summarized in Table 1 and are discussed individually in the following section.

Phenol-Degrading Bacteria (PDB)

Using the assay medium and procedure described above, PDB were detected in water samples collected from all stations during the two-day survey. Since in most cases inappropriate sample dilutions were analyzed, no definite numerical estimate of PDB could be determined. However, the densities of PDB were generally high (> 11,000/100 ml) at all stations with two exceptions. These

were observed at stations U-3 (Pilot Plant Cooling Tower) and U-8/9 (Uniroyal Building 25 N & S Cooling Tower) where PDB populations were estimated to be 4600/100 ml.

The tentative method used for the detection of PDB is based on the assumption that growth and concomitant change in color (from red to yellow) due to shift in pH (through acid production) in the assay medium indicate possible utilization of phenol. The procedure, however, does not provide any information regarding the degradation of phenol over time under the experimental conditions. Therefore, it was decided to determine phenol concentration in the inoculated and unincoculated assay medium by chemical analysis. For the purpose of this experiment, uninoculated (control) tubes of the medium and tubes inoculated with water samples (from stations CA-6, E-STP and CA-7) that showed either positive or negative reactions were subjected to chemical analysis.* In addition, the assay medium minus (without) phenol was also examined.

The results of this experiment are presented in Tale 2. In general, the concentration of phenol in each pair of the uninoculated - inoculated tubes were nearly similar and ranged from 180,000 to 197,500 ppb. However, in one instance, the assay medium tube which showed positive reaction following inoculation with water from the station CA-7 yielded a substantially lower concentration of phenol. The phenol concentration in this tube was 95,000 ppb, as compared to 192,500 ppb in the corresponding uninoculated control tube. This indicates that after inoculation, approximately 50% of phenol was degraded as determined by chemical analysis. In this particular case, this observation provides some support to the results of microbiological analysis which indicated possible phenol utilization by PDB present in water samples from CA-7.

* These were performed by the Water Quality Section of the MOE's Laboratory Services Branch. The details of procedures used are outlined in Ministry of the Environment Handbook of Analytical Methods for Environmental Samples, Vol. 2.

Chemical analysis of the assay medium, not supplemented with phenol, showed that it contained 625 ppb phenol which may have come from the phenol red indicator used to ascertain the change in color of the medium. It should be pointed out that the medium without added phenol, when inoculated with water samples, exhibits no change in color upon incubation for up to 4 days, although bacterial growth occurs (personal communication with M. Young, Taxonomy Laboratory).

Fluorescent-Pseudomonads (FP)

Since it is well established that fluorescent pseudomonads are capable of metabolizing phenols and other related aromatic compounds, as a "sole" carbon and energy source, their levels were determined using modified Drake's medium. The FP were found in water samples from all stations and their densities ranged from 21 to 240/100 ml (Table 1). The levels of FP were generally constant at most of the stations, however, appreciable variation was noticed at stations U-8/9, D/S-UD, E-STP and CA-8 during the two-day survey. Although the data are insufficient, the observed fluctuating levels of FP at these stations are probably due to the unpredictable nature and composition of the STP/Uniroyal discharges. It is interesting to note that maximum (240/100 ml) populations of FP were detected at CA-6 which is located on the Canagagigue Creek upstream of the Uniroyal plant. These elevated levels may be due to some unknown inputs from the surrounding land use activities.

Heterotrophic Bacteria (HB)

Heterotrophic bacteria are useful indicators of the degree of enrichment and trophic conditions since their concentrations are related to nutrient levels in a given body of water. The populations of HB were high at most of the stations but showed great variation in densities which ranged from 1000 to 986,000/ml (Table 1). The lowest levels were detected in samples from the Uniroyal Building (at U-8/9) and the maximum density was observed in the Shirtfactory Creek, upstream Uniroyal discharges (at U/S-UD). At all stations

located on the Canagagigue Creek downstream of the Uniroyal Plant, the HB concentrations were strikingly high and indicate substantial nutrient enrichment.

DISCUSSION AND CONCLUSIONS

The detection and enumeration of PDB by conventional methods is generally difficult and mainly depends on conjecture. The procedures commonly used require plating of samples on solid (agar) media, transfer of representative colonies to phenol enriched liquid media and measurement of undegraded phenol by chemical tests following 24-72 h incubation. Ralston and Vela (1974) developed a rapid test for the detection of phenol-degrading bacteria using a medium containing phenol. It was based on the assumption that bacteria capable of metabolizing phenol would use it as a sole source of carbon and energy for growth. Degradation of phenol in the assay medium is indicated by growth and change in pH. Although these investigators confirmed the phenol degraders population estimates (obtained with the assay medium) by chemical analysis, but the latter was not considered mandatory to the procedure. According to them, "Since methods now in use for the detection of phenol-degrading bacteria require that residual phenol be chemically assayed, it is obvious that the demonstation of a change of pH in a culture medium is a far more expedient procedure".

The method used in this investigation is a modification of Ralston and Vela's (1974) procedure. Tubes of the assay medium when inoculated with water samples (collected from various locations in the Canagagigue Creek) exhibited a distinct color change upon incubation at $35^{o}C$ for 4 days. The color change (red to yellow) was due to bacterial growth resulting in enough acid production to lower the pH of the assay medium. This indicated the potential presence of phenol utilizing bacteria. It is difficult, however, to ascertain the levels of phenols degraded and the role of PDB in abating water pollution problem caused by phenolic wastes. Nevertheless, it is generally assumed that the problem of phenol

contamination is less significant in waters which contain large populations of PDB (Stafford and Callely 1973).

Whereas the assay procedure used in this study demonstrated the potential presence of PDB, it does not provide any information regarding residual phenol concentration. Chemical analysis of representative tubes, both uninoculated and inoculated (with positive and negative reactions), revealed that very little phenol had been degraded after 4 days incubation under aerobic experimental conditions. Conversely, chemical assay also showed that in one of the four positive tubes approximately 50% of phenol was decomposed (Table 2). It is probable that bacteria, following acclimation to the initial concentration of phenolic substrate, are able to utilize it for growth and produce sufficient acid to lower pH, but the complete disappearance of phenols requires additional incubation. Some support to this argument is provided by observations made by Healy and Young (1978) who examined the phenol and catechol degradation by methanogenic bacteria under anaerobic conditions. They found that phenol degradation began after 2.5 week acclimation and an additional 14-day period was required for complete decomposition of this substrate. Similar pattern was reported by these investigators for the utilization and complete degradation of catechol.

As a general rule, phenol decompositions in polluted waters occur at temperatures above $10^{o}C$ and provided the initial concentration is not toxic (< 2 g/l) to the microorganisms (Iturriaga and Rheinheimer 1972). Bacteria which decompose phenols and other aromatic compounds belong to the families Coccaceae, Spirillaceae, Mycobacteriaceae, Pseudomonadceae, Bacteriaceae and Bacillaceae. Among fungi, yeast genera like Saccharomyces, Candida, Pichia and Debaromyces can utilize catechol as sole carbon and energy source, while molds like Neurospora, Aspergillus, Penicillium and a variety of wood-rotting fungi attack lignin, phenolics and other aromatic compounds (Evans 1963).

In any event, in the present investigation, the results of microbiological assay for PDB were not satisfactorily confirmed by chemical analysis. Therefore, no definite levels and distribution patterns of PDB could be established for the body of water examined.

Among aerobic pseudomonads, taxonomically the most complex group is the fluorescent group, in which Stainer et al. (1966) recognized three species: Pseudomonas aeruginosa, P. fluorescence and P. putida. The fluorescent pseudomonads (FP) have been found to be capable of utilizing cresol, catechol, phenols and other related aromatic compounds (Fiest and Hegeman 1969, Stainer et al. 1966). The aromatic substrates are metabolized through two different pathways (viz. ortho and meta) by these organisms as described by Evans (1963) and Fiest and Hegeman (1969). Fluctuating levels of FP were found in all water samples examined during this investigation. However, since no further taxonomic and physiological tests were performed and also due to the ubiquity of FP, it is difficult to ascertain their role and significance for phenol degradation in the water body surveyed.

At most of the sampling locations, particularly those situated downstream of the Uniroyal Plant, the densities of HB were generally high. The observed elevated levels of HB indicate poor bacterial water quality and are suggestive of eutrophic conditions in these waters.

Though the present investigation was of limited scope, the following conclusion can be drawn from the analysis of collected data:

1. The presence of FP and strikingly high concentrations of HB indicate generally poor bacterial water quality in the body of water examined.
2. Potential presence of PDB was demonstrated in the culture medium following incubation, with water samples, and 4-day incubation at $35^{o}C$. However, with respect to the actual degradation of phenolics, the microbiological observations are only partially supported by the results of chemical analysis. Therefore, the assay method used in this study appears to be unsatisfactory at the present time. As a result no accurate numerical estimates of populations

of phenol degraders could be definitely ascertained. Additional research is required before this procedure can be considered as a reliable method for the detection and enumeration of phenol-degrading bacteria in natural waters.

ADDITIONAL RESEARCH

During July - August 1978, additional work on the assay method for the detection of phenol-degrading bacteria (PDB) was carried out in the Taxonomy Laboratory of the Microbiology Section. Using water samples from Hamilton Harbour in a series of experiments, the following were examined:

(i) effect of the type and presence of indicator dye, in the assay medium, on bacterial growth, acid production and pH change;

(ii) effect of the presence or absence of phenol, in the assay medium, on bacterial growth, acid production, pH and color change and phenol degradation.

The detailed results of these experiments will be presented elsewhere, however, some of the highlights are discussed in the following section.

As summarized in the table below, following inoculation and four days incubation at 35°C, bacterial growth occurred in the assay medium (without phenol) containing either bromothymol blue, phenol red or no indicator dye. The small change in pH observed was similar to that of the sterile controls and no color change was noticed. Furthermore, a wide variety of bacteria grew in all inoculated media which contained no phenol. No bacterial growth was observed in any of the control media inoculated with sterile water.

After inoculation and four days incubation at 35°C, bacterial growth occurred in the assay medium containing phenol and either bromothymol blue, phenol red or no indicator dye. As a result of bacterial growth, enough acid was produced to lower the pH of the medium from 8.0 to c.6.2. A definite color change

was observed in the inoculated assay medium containing phenol and either of the two indicator dyes. However, the color change was easier to ascertain in the medium containing phenol red than that which contained bromothymol blue. Only two or three types of bacteria grew in the medium in the presence of phenol. This indicates that phenol inhibits certain bacteria but permits the growth of those bacteria which can utilize phenol as a sole source of carbon. Furthermore, phenol degradation in all media containing phenol was confirmed by chemical assay though the amount of phenol utilized varied from one treatment to the other. No bacterial growth was observed in any of the control media.

SUMMARY TABLE

TREATMENT	OBSERVATIONS				
	Bacterial Growth	Acid Production	pH Change	Color Change	Phenol Degradation by Chemical Assay
Assay Medium	+	-	-	NA	NA
Assay Medium + Bromothymol Blue	+	-	-	-	NA
Assay Medium + Phenol Red	+	-	-	-	NA
Assay Medium + Phenol	+	+	+	NA	+
Assay Medium + Bromothymol Blue + Phenol	+	+	+	+	+
Assay Medium + Phenol Red + Phenol	+	+	+	+	+

NA = Not Applicable, + = Positive, - = Negative.

In conclusion, the incorporation of phenol is necessary for acid production which causes a pH shift and thus a color change in the inoculated assay medium. Moreover, the demonstration of a change of pH and color is indicative of phenol degradation (as confirmed by chemical assay) and hence the presence of phenol-degrading bacteria.

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FIGURE 1. Location of Sampling Stations in Canagagigue Creek

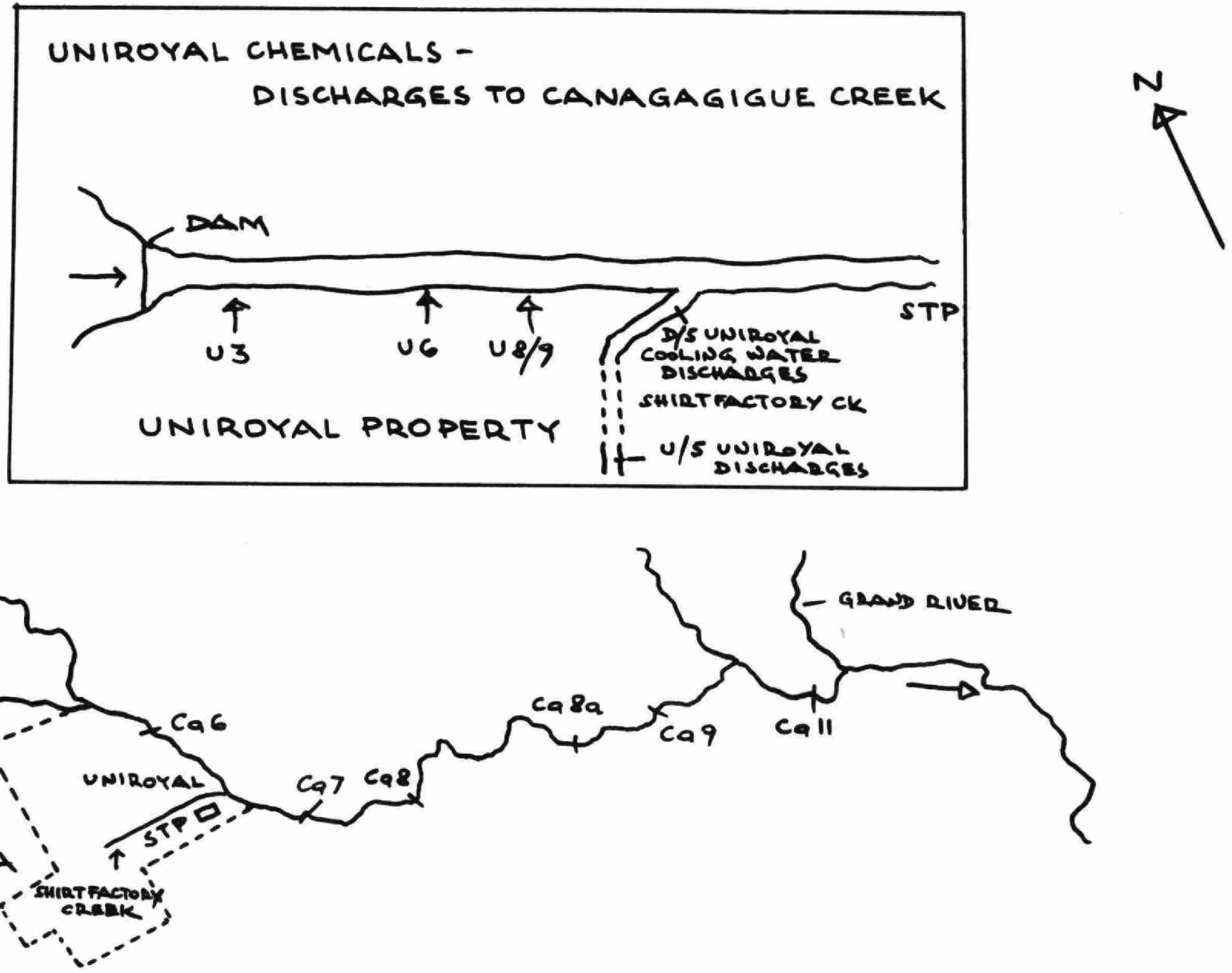

TABLE 1. SAMPLING SITES AND DENSITIES OF FLUORESCENT PSEUDOMONADS, PHENOL-DEGRADING AND HETEROTROPHIC BACTERIA IN CANAGAGIGUE CREEK

STATION	LOCATION	DATE	BACTERIAL PARAMETER PER 100 ML: PHENOL-DEGRADING BACTERIA	FLUORESCENT PSEUDOMONADS	HETEROTROPHIC BACTERIA PER ML
CA-6	Canagagigue Creek - Upstream Uniroyal Plant	24-8-77	> 11,000	240	21,130
		25-8-77	> 11,000	230	32,600
U-3	Pilot Plant Cooling Tower	24-8-77	> 11,000	93	184,100
		25-8-77	4,600	92	130,300
U-8/9	Uniroyal Building 25 N & S Cooling Tower	24-8-77	4,600	93	1,000
		25-8-77	> 11,000	36	1,060
U/S-UD	Upstream Uniroyal Discharges (Shirtfactory Creek)	24-8-77	> 11,000	93	222,000
		25-8-77	> 11,000	92	986,000
D/S-UD	Downstream Uniroyal Discharges (Shirtfactory Creek)	24-8-77	> 11,000	21	107,000
		25-8-77	> 11,000	92	88,000
E-STP	Elmira Sewage Treatment Plant (STP)	24-8-77	> 11,000	93	221,300
		25-8-77	> 11,000	230	14,100
CA-7	Canagagigue Creek (Farmers Field)	24-8-77	> 11,000	93	45,000
		25-8-77	> 11,000	92	17,600
CA-8	Canagagigue Creek (1st Bridge D/S STP)	24-8-77	> 11,000	93	23,230
		25-8-77	> 11,000	36	17,300
CA-8a	Canagagigue Creek (Farmers Bush)	24-8-77	> 11,000	75	64,300
		25-8-77	> 11,000	74	11,060
CA-9	Canagagigue Creek (Regional Rd. 22)	24-8-77	> 11,000	75	18,200
		25-8-77	> 11,000	92	14,300
CA-11	Canagagigue Creek (1st Bridge U/S Grand River)	24-8-77	SNC*	SNC	SNC
		25-8-77	2,400	150	9,300

* Sample not collected.

TABLE 2. DETERMINATION OF PHENOL CONCENTRATION IN THE UNINOCULATED AND INOCULATED ASSAY MEDIUM BY CHEMICAL ANALYSIS

STATION	TUBE NUMBER	TREATMENT	CONCENTRATION OF PHENOL ppb
CA-6	4	Uninoculated (Control)	190,000
	8	Inoculated (Positive)	190,000
E-STP	11	Uninoculated (Control)	192,500
	15	Inoculated (Positive)	192,500
	12	Uninoculated (Control)	195,000
	16	Inoculated (Negative)	195,000
CA-7	18	Uninoculated (Control)	180,000
	22	Inoculated (Weakly Positive)	182,500
	19	Inoculated (Control)	192,500
	23	Inoculated (Positive)	95,000
	20	Uninoculated (Control)	197,500
	24	Inoculated (Negative)	197,500
		Assay Medium Control (Without Phenol)	625